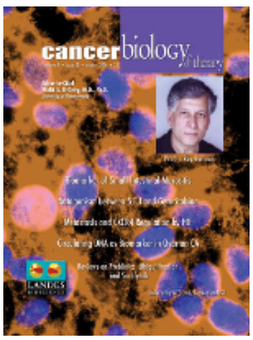

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Research Paper

Antagonistic Interactions Between Gemcitabine and 5-Fluorouracil in the Human Pancreatic Carcinoma Cell Line Capan-2

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KEY WORDS

gemcitabine, 5-fluorouracil, pancreatic carcinoma, apoptosis, bcl-2, bcl-x, survivin, NFκB, telomerase

ABBREVIATIONS

GEM	gemcitabine
5-FU	5-fluorouracil
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
PI	propidium iodide
PBS	phosphate-buffered saline
FITC	fluorescein isothiocyanate
RT-PCR	reverse transcription-polymerase chain reaction
EMSA	electrophoretic mobility shift assay
TRAP	telomeric repeat amplification protocol
IAP	inhibitor of apoptosis protein
VEGF	vascular endothelial growth factor
MMP	matrix metalloprotease
ICAM-1	intercellular adhesion molecule-1
hTERT	human telomerase reverse transcriptase

ABSTRACT

Although the recently-developed Gemcitabine (GEM) has renewed interest in clinical research in pancreatic carcinoma, it offers modest improvement of tumor-related symptoms and marginal survival advantage, even when combined with other currently-available chemotherapeutic agents such as 5-Fluorouracil (5-FU). We hypothesized that this disappointing result could be due to an interaction between the two drugs affecting cytotoxic activity. We measured in-vitro growth inhibition, cell cycle distribution, gene and protein expression of apoptosis regulators bcl-2, bcl-x and survivin, NFκB and telomerase activities of human pancreatic carcinoma cell line Capan-2 following exposure to GEM and 5-FU singly or combined, by MTT assay and median effect analysis, flow cytometry, real-time RT-PCR, Western blotting, electrophoretic mobility shift assay (EMSA) and telomeric repeat amplification protocol (TRAP) assay, respectively. We found cell growth to be inhibited by both drugs, decreasing the percentage of cells in S and G_2/M phases and inducing apoptosis, dependent on the levels of bcl-2, bcl-x_L and survivin expression in the case of 5-FU, but not for GEM. Moreover, while telomerase activity was reduced equally by both drugs, 5-FU but not GEM effectively downregulated NFκB binding activity. Intriguingly, a substantial antagonistic effect was noticed when GEM was combined with 5-FU in the concentration range tested, with the exception of the TRAP assay. These indications of an antagonistic interaction between GEM and 5-FU in some pancreatic cancer context urge further investigation of both genetic and non-genetic differences to identify the variables most relevant for optimal selection and dosing of treatment for the individual patient.

INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer deaths in Western countries and has the poorest survival of common cancers.[1] Strategies for early detection of pancreatic cancer have not yet been developed, and most patients are diagnosed with locally advanced disease or visceral metastases.[2] Therapeutic options for patients with advanced disease are few, as chemotherapy and radiotherapy are largely ineffective.[3] Metastatic disease often develops after potentially curative surgery.[4] Incidence rates are thus virtually identical with mortality rates.

Although a mammoth effort has been made to test new active drugs against this cancer, the results obtained to date with cytotoxic chemotherapy have been very disappointing.

Approximately fifty years after its synthesis,[5] 5-Fluorouracil (5-FU), a pyrimidine antagonist, is still one of the most widely used agents in the first-line therapy of pancreatic carcinoma with objective response rates below 10% and without any impact on quality of life or survival.[6] 5-FU-containing polychemotherapeutic regimens failed to yield better results.[7] More recently, the greatest change has been the acceptance of Gemcitabine (GEM), a deoxycytidine analogue with structural and metabolic similarities to cytarabine, as the standard of care for metastatic pancreatic cancer. However, GEM has demonstrated limited measurable antitumor efficacy, with objective response rates of less than 10% and median survival below 6 months.[8,9] In order to improve this dismal picture, GEM has been combined with 5-FU but collective data from several clinical trials show that combination regimens do not improve median survival of patients with advanced pancreatic carcinoma compared with single agents.[10-14]

The use of combination chemotherapy is the accepted standard for most human malignancies but little attention has been paid to drug interactions. The mechanisms of action of 5-FU and GEM as single agents or in association in pancreatic carcinoma have not yet been elucidated in depth. Here, we examine the effects of GEM and 5-FU alone or in combination on cell growth, apoptosis, expression of apoptosis-related proteins, NFκB and telomerase activity in the well-established pancreatic carcinoma cell line

Capan-2. The results of the study provide strong molecular evidence in support of the hypothesis that GEM works as an antagonistic agent in combination with 5-FU.

MATERIALS AND METHODS

Cytostatic drugs. The following chemotherapeutic agents were used: Gemcitabine (Gemzar®, Lilly Italia, Sesto Fiorentino, Italy) and 5-FU (FLUOROURACILE TEVA®, Teva Pharma Italia Srl, Milan, Italy).

Human pancreatic cell line. Human pancreatic carcinoma cell line Capan-2 (American Type Culture Collection, Rockville, MD) was grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY). The cell line was routinely screened for mycoplasm contamination using the Hoechst dye H33258 (Sigma Aldrich, St. Louis, MO).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay of cytotoxic activity. Cell were plated in triplicate in 96-well plates at a density of 5 x 10^3 cells per well in RPMI 1640 containing 10% FCS and exposed to 5-FU and/or GEM at different concentrations for time periods ranging between 24 and 72 h. During the last 4 h of incubation, 10 µl of a 5 mg/ml stock of MTT (Sigma Aldrich) was added and incubated. After addition of acid isopropanol (0.04 N HCl in isopropanol), adsorbance was determined at a wavelength of 570 nm. Percent growth relative to untreated controls was calculated based on the MTT readout and IC_{50} values were defined as the concentration of drug that produced 50% reduction in control adsorbance. Synergy or antagonism were determined using CalcuSyn analyses, based on the multiple drug effect equation of Chou and Talalay[15] and quantified by the combination index (CI). CI = 1 indicates an additive effect; <1, synergy; >1, antagonism.

Flow cytometry analysis of cell cycle distribution. To evaluate cell cycle distribution, cells were exposed to drugs as single agents or in association for various time periods as indicated. Cells detached from the culture flasks were harvested by centrifugation from the supernatants and combined with non-detached cells harvested by incubation with trypsin for 3 min at 37°C. Cell preparations were fixed with 70% ice-cold ethanol. Approximately 1 x 10^6 cells per condition were stained with propidium iodide (PI) (20 µg/ml in phosphate-buffered saline [PBS], containing 200 µg/ml RNAase A), washed and subjected to flow cytometric analysis of DNA content using a Coulter Epics IV Cytometer (Beckman Coulter, Inc., Fullerton, CA). The percentage of cells with hypodiploid DNA content (sub-G_1 fraction) was calculated by Multicycle software (Beckman Coulter).

Apoptosis detection by propidium iodide and annexin V dual staining. To determine the extent of apoptosis, cells were exposed to drugs as single agents or in association for various time periods as indicated, harvested by trypsinization for 5 min at 37°C and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI using Annexin V-FITC kit (Immunotech, Marseille, France), according the following procedure: cells were washed twice with cold PBS, resuspended (5 x 10^5 cells in 100 µl assay binding buffer) and incubated with 1 µl Annexin V-FITC and 5 µl propidium iodide (PI) for 10 min in the dark on ice. Binding buffer (400 µl) was then added and cells were analyzed by flow cytometry. Ten thousand cells were characterized for apoptosis.

Table 1 **Primer sequences for mRNA quantification by real-time RT-PCR**

Primer set	GenBank Accession #	Primer sequence (5' → 3')	RT-PCR *E* (%)
β-actin sense β-actin antisense	NM_001101	GCG AGA AGA TGA CCC AGA TC GGA TAG CAC AGC CTG GAT AG	98
bcl-2 sense bcl-2 antisense	NM_000633	AGT TCG GTG GGG TCA TGT GTG CTT CAG AGA CAG CCA GGA GAA ATC	110
bcl-x_L sense bcl-x_L antisense	Z23115	GCA GGT ATT GGT GAG TCG GAT CGC CAC AAA AGT ATC CCA GCC GCC G	95.6
survivin sense survivin antisense	AF077350	ATT CGT CCG GTT GCG CTT TCC CAC GGC GCA CTT TCT TCG CAG	91.9

E = efficiency deducted from the slope (s) of the standard curve based on $E = e^{\ln 10/-s} - 1$

RNA isolation and reverse transcription (RT). Total RNA was isolated from Capan-2 cells and appropriate positive controls, using Trizol (Invitrogen, Life Technologies, Gaithersburg MD) following the manufacturer's instructions. To remove traces of genomic DNA, total RNAs (1 µg) were treated with DNase I (Invitrogen) and reverse-transcribed to cDNAs using SuperScript II (Invitrogen) as described elsewhere.[16]

RT-polymerase chain reaction (PCR) analysis. For each PCR, 10 µl of first-strand cDNA were added to 20 µl of PCR mix containing 1 U Taq polymerase and 100 ng each of 5' and 3' primers and 100 ng of internal standard gene upstream and downstream primers (β-actin) to minimize variations in amplification efficiency between tubes. All PCR reagents were purchased from Life Technologies. PCR products were analyzed by size fractionation, using 2% agarose gels stained with ethidium bromide. β-actin primers, amplification conditions and PCR product sizes have been described by us previously.[16] Human *bcl-x* primers were: 5'-TTG GAC AAT GGA CTG GTT GA-3' (sense) and 5'-GTA GAG TGG ATG GTC AGT G-3' (antisense). The sequences of sense and antisense primers used for *bcl-x* were designed to detect both long (*bcl-*x_L) and short (*bcl-*x_S) form mRNAs.[17] The PCR protocol was as follows: 35 cycles of 94°C/1 min for denaturation, 60°C/1 min for annealing and 72°C/2 min for extension. The predicted size of *bcl-*x_L- and *bcl-*x_S-PCR products were 780 bp and 591 bp, respectively.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR analysis was performed on iCycler iQ system (Bio-Rad, Hercules, CA) via SYBR green I dye detection. β-*actin, bcl-2, bcl-*x_L and *survivin* were amplified in duplicate on PCR optical 96-well reaction plates (Bio-Rad). 25 µl of the PCR mixture in each well contained 5 µl of cDNA (corresponding to 100 ng of total RNA), 2.5 µl of each sequence-specific primer (150 nM for β-actin, 300 nM for *bcl-*x_L, 200 nM for *survivin* and *bcl-2*), 12.5 µl of 1X iQ SYBR Green Supermix (Bio-Rad) and 2.5 µl of nuclease-free water. Primer sequences were designed to be cDNA specific and to work under equivalent reaction conditions using Beacon Designer 2 Software (Bio-Rad). Primer sequences and reaction efficiency are listed in Table 1. A negative PCR control without cDNA template and a positive control sample with a known Ct value were included in each assay. Optimized thermal cycling conditions were as follow: 5 min at 95°C, then 40 cycles of a 15-second melt at 95°C followed by a

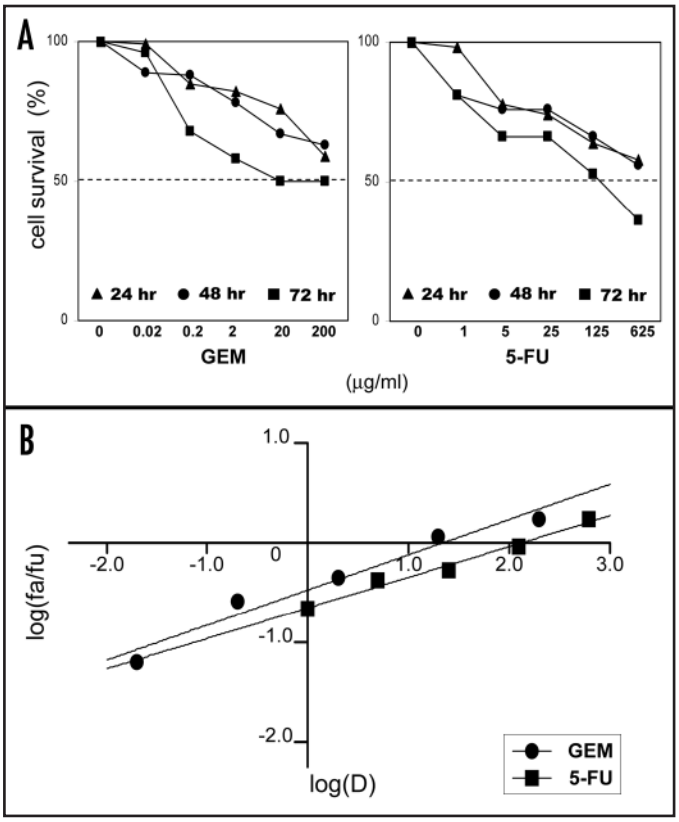

Figure 1. (A) Dose-response curves of GEM and 5-FU individual treatment in Capan-2 cells. Cells were treated with drugs for 24, 48 and 72 h and cytotoxicity was analyzed by growth inhibition assay using MTT reagent. Cytotoxicity assay was repeated three times with three replications in each experiment and mean surviving cells (% of control) were plotted against drug concentrations. (B) Median-effect plot of cytotoxicity data of 72 h treatment developed by CalcuSyn software. The median-effect plot is based on logarithmic form of Chou and Talalay's equation, as described in Materials and Methods section. The median-plot is a plot of $x = \log(D)$ versus $y = \log(fa/fu)$ where D is the dose of the drug, fa is the fraction affected by the dose and fu is the fraction not affected by the dose. Based on this equation, if median-effect dose and slope of the median-effect plot are known, dose of the drug in individual treatment corresponding to any affected fraction can be determined.

1 minute annealing/extension at 60°C (two step PCR). Specificity of the PCR products was confirmed by the melting curve program at the end of the reaction (55°C to 95°C with a heating rate of 0.5°C/10 seconds and continuous fluorescence measurements). PCR efficiency (*E*) was determined using the iCycler iQ software and the method described by Ramakers et al.[18] For each sample the cycle threshold value (Ct) was acquired using the Fit point Method.[19] The relative expression ratio of the target genes was computed using the Relative Expression Software Tool (REST).[20] This software calculates an expression ratio relative to the control (untreated Capan-2 cells) normalized by a reference gene (β-*actin*). The expression ratio (R) is:

$$R = E_{target}^{\Delta Ct \text{ target (mean control-mean sample)}} / E_{reference}^{\Delta Ct \text{ reference (mean control-mean sample)}}.$$

Western blotting. Immunoblotting of bcl-2, bcl-$x_{L/S}$, survivin and IκB-α proteins contained in cell lysates was performed as described.[17] After 48 h and 72 h treatment with chemotherapeutic agents, Capan-2 cells (1 x 10^6) were suspended in 200 μl of RIPA buffer (150 mM, 50mM Tris HCl pH 7.5, 1% Non-idet P-40, 0.5% sodium dexycholate, 0.1% SDS) supplemented with 10 μg/ml aprotinin, 15 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (all from Sigma Aldrich) on ice for 30 minutes. After removal of cell debris by centrifugation, the protein concentration in cell lysates was determined using a Bradford protein assay (Biorad). Equal amounts of protein (40 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane (Biorad). Membranes were blocked over-night in blocking buffer (5% non-fat dry milk in TBS containing 0.1% Tween-20). The membranes were then probed with appropriate concentrations of a rabbit polyclonal antibody to bcl-2 (Transduction Laboratories, Lexington, KY), a rabbit polyclonal antibody to bcl-$x_{L/S}$ (Transduction Laboratories), a rabbit polyclonal antibody to survivin (Novus Biologicals, Littleton CO) and, a rabbit polyclonal antibody to IκB-α (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, membranes were incubated for 1 h with the appropriate secondary antibody-peroxidase conjugate (1:3500 dilution, Amersham, Arlington Heights, IL). Following several washes, the blots were developed by chemiluminescence followed by autoradiography (ECL, Amersham Corp., Airlngton Heights, IL). Band intensity was quantified by densitometric analysis using Kodak 1D Image Analysis Software (Eastman Kodak Company, New Haven, CT).

Nuclear extract preparation and electrophoretic mobility shift Assay (EMSA). Capan-2 cells were treated with IC_{50} doses of GEM and/or 5-FU for 48 h and nuclear extracts were prepared by the method elsewhere described.[21] EMSA were performed by incubation of 0.5 ng of ^{32}P-labeled double stranded NFκB oligonucleotides (5' AGT TGA GGG GAC TTT CCC AGG C-3', Santa Cruz Biotechnologies) with 5 μg of nuclear extract in a binding buffer 75 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, 1 mM DTT and 10% glycerol containing 1 μg of poly (dI-dC) for 20 min at room temperature. The reaction were analyzed on 6% polyacrylamide gels in 0.5X TBE (tris/borate/EDTA) buffer.

Telomeric repeat amplification protocol (TRAP)-ELISA Assay. For detection of telomerase activity, a photometric enzyme immunoassay using the telomeric repeat amplification protocol was used (TeloTAGGG Telomerase PCR ELISA^{PLUS}, Roche, Rotkreuz, Switzerland). The test was performed following the manufacturer's instructions with some modifications. Briefly, 0.2 μg of cellular protein were used for primer elongation/amplification reaction and 2.5 μl of the amplification product was transferred for hybridization and the ELISA procedure. Telomerase activity was calculated as suggested in the kit's manual and compared with a control template of 0.1 mol/ml telomeric repeats, representing a relative telomerase activity (RTA) of 100. Each assay contained heat inactivated samples and lysis buffer as negative controls.

Statistical analysis. Levels of statistical significance were evaluated with data from at least three independent experiments by the Student's-t-test using SigmaStat (Systat Software, Point Richmond, CA). $p < 0.05$ was considered statistically significant.

RESULTS

Growth inhibitory effects of GEM and 5-FU on Capan-2 cells. To establish the growth inhibitory effects of GEM and 5-FU, the

Table 2 **Computer simulated CI values for GEM and 5-FU at 50%, 75% and 90% inhibition of Capan-2 cells[a]**

Inhibition (%)	CI[b] ± est. SD
50	2.08± 1.22 (antagonism)
75	1.59±1.48 (antagonism)
90	1.23±1.73 (moderate antagonism)

[a]The values were determined using CalcuSyn software. [b]Combination index at combination ratio of GEM and 5-FU (1:5). CI < 1, =1 and >1 indicate synergism, additive effect and antagonism, respectively.

Capan-2 cells were first cultured in the presence of GEM (range from 0.02 to 200 μg/ml) and 5-FU as single agent (range 0 to 625 μg/ml) for 24, 48 and 72 h. As shown in (Fig. 1A), while 5-FU exhibited dose-time-dependent cytostatic/cytotoxic activity, the effect of GEM was only partially dose- dependent, since increasing the concentration from 20 to 200 μg/ml did not significantly increase the effect of the drug. However, median-effect analysis (Fig. 1B) demonstrated that GEM is a more potent cytotoxic agent that 5-FU in Capan-2 cells, as expressed by the computed potency values (*Dm* [IC_{50}]: 20 μg/ml for GEM versus 125 μg/ml for 5-FU) after 72 h treatment and slope coefficient of the curve (*m*: 0.34 ± 0.04 for GEM and 0.31 ± 0.02 for 5-FU), with a high conformity coefficient for both drugs (r: 0.98 and 0.99, respectively).

Interactions between GEM and 5-FU affecting growth of Capan-2 cells. Using the median-effect plot analysis of Chou and Talalay,[15] we assessed whether the GEM plus 5-FU combination produced synergistic, additive, or antagonistic effects on the growth of Capan-2 cells. In this analysis, Capan-2 cells were exposed for 72 h to serial dilutions from the lowest concentration of GEM plus 5-FU combined, maintaining a fixed ratio of 1:5 based on the IC_{50} values obtained for each drug alone. Table 2 summarizes the quantitative measure of the degree of drug interaction in terms of effect for a given endpoint, and shows that CI values for IC_{50}, IC_{75} and IC_{90} estimates were all above the additive CI of 1, indicating an antagonistic effects of GEM and 5-FU when simultaneously administered.

Effects of GEM and 5-FU alone or in combination on cell cycle distribution of Capan-2 cells. To assess whether inhibition of metabolic rates by GEM and 5-FU, alone or in combination, as observed in MTT assays, was due to inhibition of cell cycle progression or to induction of apoptosis, we analyzed the effects of the drugs on cell cycle distribution, by flow cytometry. A representative experiment is shown in Figure 2. Both GEM and 5-FU treatments had significant measurable effects on cell-cycle distribution of Capan-2 cells (Table 3). In particular, the percentage of cells in both S and G_2/M phases of the cell cycle significantly decreased after 72 h drug-exposure (S phase: GEM vs. control, p = 0.004, 5-FU vs. control p = 0.002; G_2/M phase: GEM vs. control, p < 0.001; 5-FU vs. control p = 0.001). In contrast, the fraction of cells with sub-G_1 DNA content consistently increased in the presence of GEM and 5-FU in a time-dependent fashion consistent with induction of apoptosis (72 h: GEM vs. control p = 0.02; 5-FU vs. control, p = 0.004). Again, the combination of GEM and 5-FU was associated with markedly lower rates of apoptosis compared to those of single drugs (34.5% ± 0.5 vs. 31.6 ± 2.5 for GEM alone, p = 0.04 and vs. 48.7 ± 2.3 % for 5-FU alone, p = 0.005) after 72 h of treatment (Table 3). Taken together these results indicate that both single drug treatments can induce apoptosis of Capan-2 cells, but in combination GEM antagonistically interacts with 5-FU.

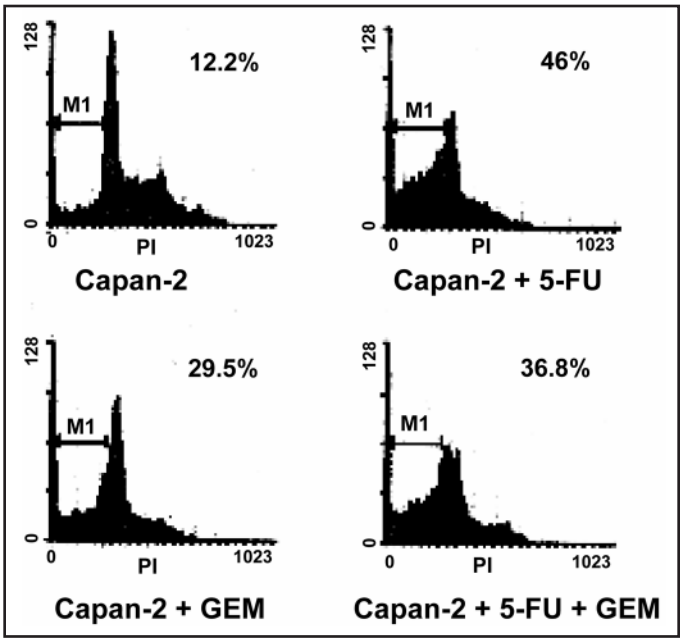

Figure 2. Cell-cycle analysis by flow cytometry of Capan-2 cells treated with GEM and 5-FU alone or in combination. Capan-2 cells were exposed to single or combined IC_{50} doses of GEM and 5-FU. The cells were fixed with 70% ice-cold ethanol, stained with propidium iodide (PI) and analyzed by flow cytometry. The percentages of cells in the G_0/G_1, S and G_2/M are detailed in Table 3. Data refer to a representative experiment after 72 h treatment performed three times with similar results.

Effects of GEM and 5-FU alone or in combination on apoptosis of Capan-2 cells. In order to further explore the increases in the sub-G_1 population following GEM and/or 5-FU treatment of Capan-2 cells, we quantified the extent of apoptosis by flow cytometric

Table 3 **Effect of GEM and 5-FU alone or in combination on cell cycle phase distribution in Capan-2 cells**

Drug concentration	G_0/G_1	S	G2/M	Apoptosis
0 (control)				
24 h[a]	36.9 ± 2.5[b]	22.8 ± 1.2	14.4 ± 0.2	12.1 ± 0.7
48 h	32 ± 1.2	26.8 ± 0.6	15.5 ± 0.2	9.6 ± 0.3
72 h	31.5 ± 0.5	20.2 ± 1.5	15.9 ± 0.3	11.7 ± 0.4
GEM (20 μg/ml)				
24 h[a]	44.5 ± 2	23.4 ± 1.1	9 ± 0.2	12.4 ± 0.3
48 h	37.4 ± .1	17.6 ± 0.9	7.6 ± 0.3	27.7 ± 0.6
72 h	26.6 ± 0.8	14.9 ± 1	6.3 ± 0.2	31.6 ± 2.5
5-FU (125 μg/ml)				
24 h[a]	47 ± 0.3	20.6 ± 0.8	6.8 ± 0.1	16 ± 0.9
48 h	23.2 ± 2	14.4 ± 1	3.4 ± 0.3	46.9 ± 2.1
72 h	3.5 ± 1.3	14.3 ± 1.2	5.2 ± 0.3	48.7 ± 2.3
GEM + 5-FU				
24 h[a]	44.3 ± 1.2	23.7 ± 0.5	9.3 ± 0.2	14 ± 0.4
48 h	28 ± 0.5	16.3 ± 0.4	6.2 ± 0.1	37.6 ± 0.8
72 h	22 ± 0.8	17.6 ± 0.2	7.3 ± 0.3	34.5 ± 0.5

[a]Incubation time in hours; [b]Values are mean percentages ± SD of three experiments.

Table 4 **Percentages of cells of viable, early apoptotic, late apoptotic/necrotic cells**

	% cells		
Culture conditions	**Viable cells[a]**	**Early Apoptosis[b]**	**Late Apoptosis/Necrosis[c]**
24 h			
Control	90.9 ± 2.8[d]	7.9 ± 2.8	1.1 ± 0.1
GEM	86 ± 5.7	12.9 ± 5.3	1.5 ± 0.3
5-FU	87.5 ± 4.3	7.9 ± 2.8	1.3 ± 0.2
GEM + 5-FU	89.1 ± 0.6	9.7 ± 0.4	1.2 ± 0.2
48 h			
Control	93.3 ± 0.4	5.4 ± 0.3	1.3 ± 0.1
GEM	83.2 ± 0. 5	15.3 ± 0.1	1. ± 0.3
p[e] *vs control*	*<0.001*	*<0.001*	
5-FU	71.5 ± 0.5	25.9 ± 0.7	2.5 ± 0.1
p vs control	*<0.001*	*<0.001*	*0.01*
p vs GEM	*<0.001*	*<0.001*	
GEM + 5-FU	73.9 ± 1.1	24.7 ± 0.6	1.3 ± 0.5
p vs control	*<0.001*	*<0.001*	
p vs GEM	*0.001*	*<0.001*	
p vs 5-FU	*0.02*	*0.002*	
72 h			
Control	89 ± 6.8	8.7 ± 5.7	2.2 ± 1
GEM	76.1 ± 1.2	19.2 ± 0.6	4.7 ± 0.6
p vs control		0.001	
5-FU	59.8 ± 5.8	32.1 ± 4.7	8 ± 1
p vs control	*<0.001*	*<0.001*	*0.01*
p vs GEM	*<0.001*	*<0.001*	*0.04*
GEM + 5-FU	65.5 ± 7.5	26 ± 4.7	6.6 ± 1.5
p vs control	*<0.001*	*<0.001*	
p vs GEM	*0.001*	*<0.001*	
p vs 5-FU	*0.02*	*0.002*	

[a]Viable cells = non-apoptotic cells (Annexin V and PI double-negative cells. [b]Early apoptotic cells = Annexin V positive and PI negative cells). [c] Late apoptotic or necrotic cells = double-positive cells or PI positive cells. [d]Mean percentage ± SD of three separate experiments. [e]*p* Only statistically significant differences are shown.

analysis of cells labelled with annexin V and PI. Phosphatidylserine externalisation is a characteristic of cells undergoing apoptosis and Annexin V has a strong affinity for phosphatidylserine. The simultaneous staining of cells with Annexin V and PI enabled us to distinguish between non-apoptotic cells (double-negative, Fig. 3, lower left quadrant), early apoptotic cells (annexin V positive and PI negative, Fig. 3, lower right quadrant), and late apoptotic or necrotic cells (double-positive or PI positive and Annexin V negative, Fig. 3, upper right quadrant and upper left quadrant, respectively). Capan-2 cells were incubated with GEM (20 μg/ml) or 5-FU (125 μg/ml) alone or in combination for 24, 48 and 72 h. Consistent with previous experiments, starting from 48 h of treatment, GEM caused a significant decrease of viable cells accompanied by an increase in the percentage of cells undergoing early apoptosis, while 5-FU resulted in an increase of the percentage of both early and late apoptotic cells. Cells treated with the GEM plus 5-FU combination showed a significant decrease in viable cells and an increase in early apoptotic cells versus those treated with GEM alone, the opposite occurred in cells treated with 5-FU alone, particularly after 72 h of treatment. (Fig. 3, Table 4). Thus it would appear that in Capan-2 cells 5-FU alone more efficiently induces substantial increase in cells undergoing apoptosis compared with either GEM or combination.

Effects of GEM and 5-FU on apoptosis regulators: bcl-2 family and survivin. These results raised the issue of how GEM and 5-FU treatment sensitizes Capan-2 cells to apoptosis induction. To address this question, we used quantitative real-time RT-PCR to analyze expression of mRNA for the anti-apoptotic proteins bcl-2, bcl-x_L and survivin in Capan-2 cells upon treatment with GEM and 5-FU, alone or in combination. Figure 4A shows that 48 h-treatment of Capan-2 cells with GEM as single agent has only a marginal negative effect on the accumulation of *survivin, bcl-2* and *bcl-x_L* transcripts. 5-FU significantly lowered the mRNA expression of *survivin, bcl-2* and *bcl-x_L* with concomitant induction of the message for the pro-apoptotic bcl-x_S isoform, as demonstrated by standard RT-PCR. In combination GEM antagonized 5-FU-induced downregulation of messages for all three the anti-apoptotic molecules. These changes in gene-expression profile correlate with a compatible change in protein-expression revealed by Western blot analysis, assessing expression of survivin, bcl-2 and bcl-$x_{L/S}$ in Capan-2 cells after exposure to GEM and 5-FU alone or in combination (Fig. 5). Expression of survivin and bcl-2 dramatically decreased in Capan-2 cells only after treatment with 5-FU for 72 h, whereas expression of bcl-x_L did so to a lesser extent. Again, in combined treatment GEM interacted antagonistically with the 5-FU induced downregulation.

Effects of GEM and 5-FU on activation of the NFκB transcription factor in Capan-2 cells. The NFκB pathway regulates numerous downstream oncogenic, apoptotic and growth-related signals and has been implicated in the growth of diverse neoplasms, including pancreatic carcinoma.[22] Thus, to determine whether GEM and 5-FU treatments can suppress NFκB activity, Capan-2 cells were cultured in the presence of IC_{50} doses of 5-FU and GEM, alone or in combination, for 48 h. In agreement with a previous report,[23] EMSA revealed constitutive binding of NFκB transcription factor in Capan-2 cells. The specific binding of NFκB to DNA could be abrogated with an excess of unlabeled probe, indicating that NFκB activity is actually contained in the cells (data not shown). As shown in Figure 6, 5-FU but not GEM treatment inhibited NFκB activation in Capan-2 cells. The association of the drugs reduces 5-FU-induced suppression of the NFκB activity. Thus, 5-FU is likely to interfere with the signalling cascade that leads to NFκB activation.

5-FU prevents IκB-α degradation. The suppressed NFκB DNA binding observed in Capan-2 cells after 5-FU treatment could be due to a prevention of the degradation of IκB-α protein and the subsequent release of the NFκB. Immunoblot analysis of cell extracts from untreated and 5-FU-treated cells revealed significant changes in protein levels of IκB-α in treated cells (Fig. 7A). Densitometric analysis of the bands demonstrated that, especially after 24 hr exposure, treatment with 5-FU induces upregulation of IκB-α protein in Capan-2 cells (Fig. 7B).

Effects of GEM and 5-FU on telomerase activity in Capan-2 cells. Telomerase activity is frequently associated with malignant phenotypes and may be considered an ubiquitous tumor marker. Because an anti-apoptotic function of telomerase has been described for other cells, and cells undergoing apoptosis are associated with decreased telomerase activity,[24] we considered the possibility that GEM and 5-FU may induce apoptosis in Capan-2 cells by down-regulating telomerase. To determine whether the drugs decrease telomerase, Capan-2 cells were treated with GEM and 5-FU alone or in combination, and telomerase activity was measured by the PCR-based telomeric TRAP. As shown in Figure 8, Capan-2 cells

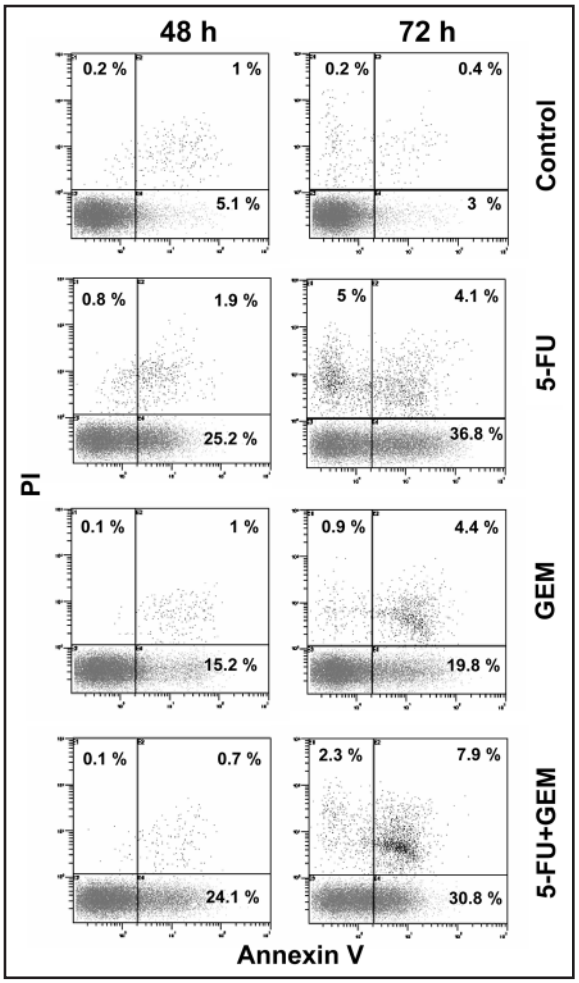

Figure 3. Flow cytometric analysis of Capan-2 cells labelled with Annexin V and PI. Capan-2 cells were incubated in the absence of cytotoxic drugs or exposed to single or combined IC_{50} doses of GEM and 5-FU for 24, 48 and 72 h. Cells were harvested, and stained with Annexin V and PI. The X-axis represents Annexin V related fluorescence and the Y-axis represents PI-related fluorescence. The percentages of cells in quadrants representative of viable, early apoptotic, late apoptotic/necrotic cells are shown in Table 4. The results are representative of three independent experiments.

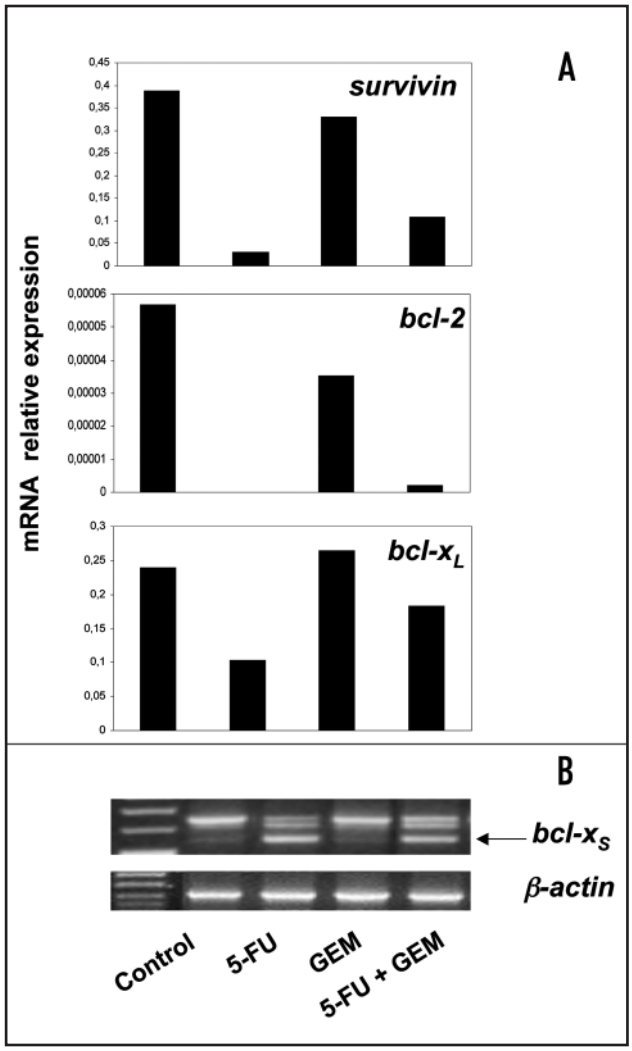

Figure 4. (A) Effect of chemotherapy on the relative expression of *survivin*, *bcl-2* and *bcl-*x_L genes. Capan-2 cells were either left untreated or treated with IC_{50} doses of 5-FU or GEM as single agents or in combination for 48 hr and analyzed for content of *survivin*, *bcl-2* and *bcl-*x_L mRNA by quantitative real-time RT-PCR. Values were normalised on the basis of β-actin expression in the corresponding samples. Values are means of determinations in duplicate. (B) Modulation of *bcl-*x_S mRNA expression in Capan-2 cells in the same conditions reported above was assessed by standard RT-PCR.

expressed a constitutive level of telomerase activity that was significantly decreased by both chemotherapeutic agents, used alone and in combination: activity was decreased to about 71% of the control value already after 24 h of treatment. Because telomerase downregulation preceded inducement of apoptosis, the data are consistent with the idea that decreased telomerase activity might contribute to cell death induced by the drugs.

DISCUSSION

After multiple attempts to improve the efficacy of 5-FU monotherapy by biochemical modulation failed,[25] GEM became the standard first-line agent in patients with advanced pancreatic cancer, a phase III trial having demonstrated a modest increase in survival accompanied by an improvement in the disease-related symptoms of pain, weight, and performance status, versus 5-FU.[8] However, subsequent phase III trials have rarely shown objective response rates exceeding 5.4% or 1-year survival rates better than 20%.[26] To improve on the results obtained with GEM alone, combinations of GEM with 5-FU have been evaluated; however, addition of 5-FU to GEM has not significantly increased the survival of patients with pancreas cancer compared with GEM alone.[27] The mechanism(s) responsible for these ineffective interactions remain elusive. This is the first in vitro demonstration that the antiproliferative and apoptotic effect of the combination of GEM and 5-FU against human pancreatic cancer cells is subadditive of the effects of the two drugs administered alone, indicating that the combination schedule usually applied for these two drugs in some pancreatic carcinoma patients (GEM and continuous infusion of 5-FU) may be neither efficacious nor advisable.

As shown by the CI values > 1 at all levels of killed cell fraction, simultaneous treatment with GEM and 5-FU caused antagonistic effects in Capan-2 cells. Similar interactions have been observed when GEM was combined with docetaxel or epidoxorubicin, in human gastric[28] and bladder cancer[29] cell lines, respectively.

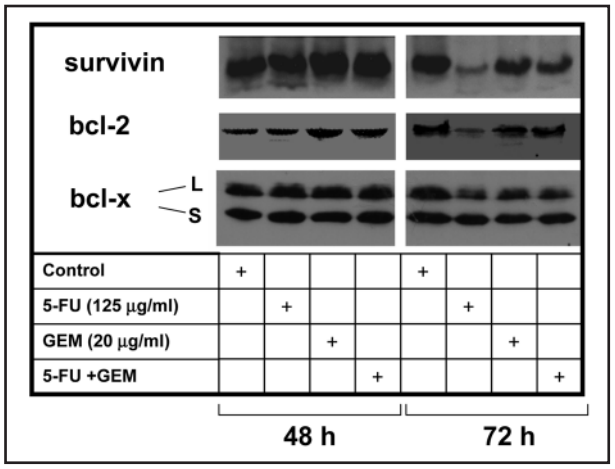

Figure 5. Western blot assay of survivin, bcl-2 and bcl-x protein expression in Capan-2 cell line. Cells were treated with IC_{50} doses of 5-FU or GEM as single agents or in combination for 48 and 72 h. Protein (40 μg/lane) from cell lysates was electrophoresed in SDS-PAGE gels, transferred to membranes, and probed with anti-survivin, anti-bcl-2 and anti-bcl-x antibodies, respectively, as described in the Material and Methods section.

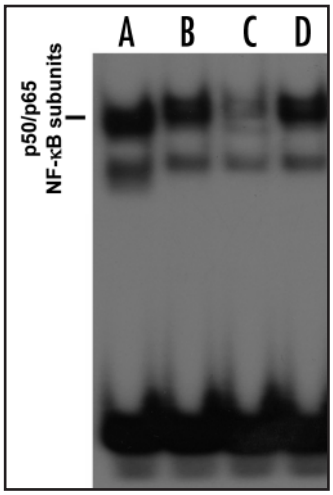

Figure 6. Constitutive and chemotherapeutic-agent-modulated NFκB binding as demonstrated by the electrophoretic mobility shift assay (EMSA) in Capan-2 cells. Cells were not exposed (A) or exposed to IC_{50} doses of GEM alone (B) 5-FU alone (C) or their combination (D) for 48 h. 5-FU inhibited NFκB binding. The same amount of protein (8 μg) was used in each lane.

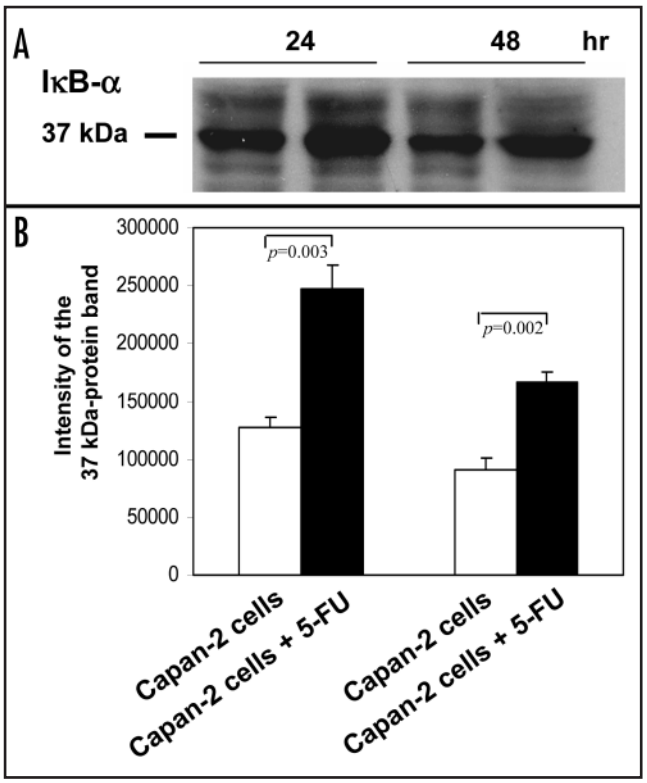

Figure 7. Effect of 5-FU treatment on IκB-α protein degradation. (A) Western blot of IκB-α protein in cytosolic extract of Capan-2 cells. The cells were not exposed or exposed to 5-FU (IC_{50} dose) for 24 and 48 h. The experiment was performed three times and a representative experiment is shown. (B) Densitometric quantification of data presented in the top panel. Mean ± SD of three different determination. *p* vs respective control.

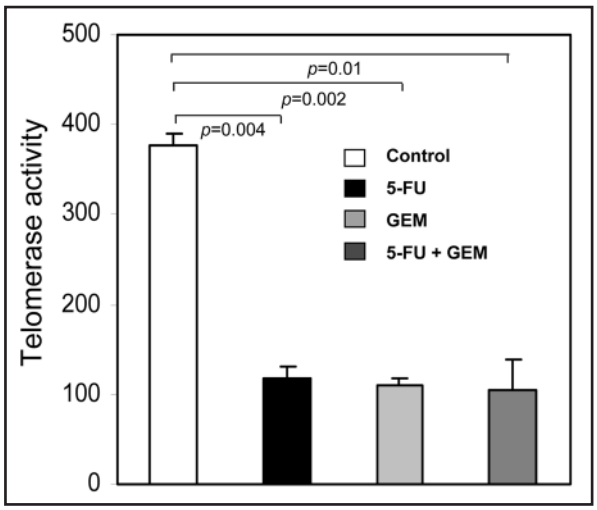

Figure 8. 5-FU and GEM decreased telomerase activity in Capan-2 cells. The Capan-2 cells were unexposed or exposed to IC_{50} doses of 5-FU and GEM alone or in combination for 24 h. Cellular extracts with equal amount of protein were subjected to the PCR-TRAP assay, as described in the Materials and Methods section. The experiment was performed three times. *p* vs respective control.

It is noteworthy that this antagonistic effect is evident only in Capan-2 cells and not in other pancreatic cell lines, such as BxPC-3 and PT45 (data not shown). Capan-2 cells carry mutations at the *k-ras* and *p16* loci, and wild type *p53*,[30] while BxPC-3 carries mutations at *p53* and *p16* loci and wild type *k-ras* and PT45 carry mutations at *k-ras* and *p53* loci and homozygous deletion at the *p16* locus.[31,32] As previously reported, p53 alterations seems not to influence the sensitivity of pancreatic cancer cells to a variety of anticancer agents, including 5-FU and GEM.[33,34]

All three cell lines carry alterations at *p16* locus that, on the contrary, appears to be involved in the sensitivity of pancreatic cancer cells to chemotherapeutic drugs.[33] We do not know which features of Capan-2 cells render them more resistant to the combination of GEM and 5-FU than to either drug singly, whereas this is not the case of other pancreatic carcinoma cells. However, our results appear to indicate that the antagonistic effects of GEM and 5-FU combined treatment are cell-type specific.

To explain the possible mechanism underlying the antagonistic interaction, we analyzed the perturbations induced by the drugs on cell cycle and apoptosis by flow cytometry, and in some of its related markers by the immunoblot technique. Both GEM and 5-FU induced a decrease in the percentage of cells in the S phase and an increase in the percentage of cells with a sub-G_1 DNA content, expressing AnnexinV and/or PI, in time-dependent manner, consistent with antiproliferative and apoptotic effects. The slight but significant antagonistic interaction of GEM with 5-FU at the DNA level, might explain the decreased growth inhibition observed when the two agents are used in combination.

It is widely accepted that apoptosis is an active gene-directed cellular suicide mechanism, and that many genes contribute to its regulation. Among these, *bcl-2* and *bcl-x* have been paid particular attention because they may be key factors in the final pathway involved in regulation of cell apoptosis.[35,36] In particular, the *bcl-x* gene gives rise to two proteins, bcl-x_L and bcl-x_S, via alternative mRNA splicing. bcl-x_L shows remarkable homology to bcl-2 and seems to inhibit apoptosis as effectively as bcl-2 in some cells. In addition, bcl-x_L has the potential to prevent cell death where bcl-2 fails to do so, suggesting that these two similar proteins control partially-independent pathways of apoptosis.[37] In contrast, the short form product of the *bcl-x* gene, *bcl-*x_S, encodes a protein with opposite effects functioning as a promoter of apoptosis.[38] The majority of human cancers are found to have overexpression of bcl-2, bcl-x_L, or both.[39] bcl-2 and bcl-x_L may play a critical role in cancer progression and resistance to a wide spectrum of chemotherapeutic agents and radiation therapy.[40]

In this study, we found that bcl-2 and bcl-$x_L$were expressed in Capan-2 cells. At the IC_{50} concentration, 5-FU exposure potently downregulated bcl-2 at both gene expression and protein levels, while bcl-x_L was less affected. However, as demonstrated by RT-PCR and Western blot, drug treatment induced mRNA expression of the pro-apoptotic short form of the gene *bcl-*x_S, with a slight increase in the relative protein. Since apoptosis is a complex process regulated by a balance between inducers and inhibitors that are simultaneously expressed, it may be assumed that the resulting net effect after 5-FU treatment could lead to a lower expression ratio of anti-apoptotic bcl-2 and bcl-x_L proteins to pro-apoptotic bcl-x_S, which might be responsible for the 5-FU-induced apoptosis in Capan-2 cells.

By contrast, GEM slightly downregulated the level of bcl-2 mRNA and protein, did not influence that of bcl-x and, when combined with 5-FU, exerted an antagonistic interaction.

In addition to the bcl-2 family, several other proteins have been identified to contribute to the inhibition of apoptotic signaling, including survivin.[41] This member of the inhibitor of apoptosis protein (IAP) family is unique in that it is a bifunctional protein that controls cell division and counteracts apoptosis downstream of the mitochondria and death receptors, by acting as endogenous inhibitors of caspases.[42] Moreover, in addition to prolonging the cell life-span, survivin enables cancer cells, such as the pancreatic ones, to suppress attacks from the immune system effectors by inhibiting Fas-mediated apoptotic signaling, and also induces apoptosis in immune cells via induction of FasL on the cancer cell surface.[43] Survivin, absent in most normal adult tissues, represents the fourth top gene expressed in cancers of the lung, colon, brain, breast and in melanoma,[44-48] and is implicated in resistance to apoptosis induction by anti-cancer agents and ionizing radiations.[49,50] It has been demonstrated that downregulation of survivin expression, using conventional antisense or siRNA, facilitates cancer cell apoptosis and sensitizes cells to anti-cancer agents.[51]

This study showed that survivin is strongly expressed in human pancreatic cancer Capan-2 cells and that 5-FU, but not GEM, strongly downregulates its expression. This downregulation is at the transcription level, since 5-FU did significantly reduce *survivin* mRNA. Again, antagonistic effect of GEM treatment on 5-FU-induced modulation of survivin was observed.

It is interesting to note that nearly all molecules that mediate apoptosis resistance in pancreatic cancer cells, such as bcl-2, bcl-x_L, and survivin, are controlled by the transcription factors of NFκB family, thus putting NFκB at center stage regarding apoptosis resistance in pancreatic cancer. The contribution of NFκB to the development and maintenance of numerous cancers has been clearly documented.[52] In pancreatic cancer cell lines, inhibition of NFκB activity enhances sensitivity to chemotherapeutic agents and death receptor-mediated apoptosis by downregulating the expression of the anti-apoptotic bcl-2 family member bcl-x_L and the caspase 8 inhibitor c-Flip.[53,54] In addition to these anti-apoptotic functions, NFκB also contributes to the control of proliferation in pancreatic cancer cells.[55]

We found that 5-FU, but not GEM, inhibited NFκB DNA-binding activity by preventing degradation of IκBα proteins, allowing active NFκB to translocate to the nucleus, which may explain both downregulation of NFκB target genes *survivin*, *bcl-2, bcl-*x_L, during 5-FU-induced cell growth inhibition, and the induction of apoptosis. Similar suppression of NFκB activity caused by 5-FU has been described in stomach and salivary-gland cancer cells.[56-58] In accordance with our results concerning the inability of GEM treatment to abrogate NFκB activity in Capan-2 cells, recent studies on other pancreatic carcinoma cell lines, both in vitro and in vivo, showed that GEM alone not only is ineffective in inhibiting NFκB binding activity, but may even activate NFκB, suggesting a potential mechanism of acquired chemoresistance.[59]

Our observations are of interest, since suppression of NFκB activity may also involve transcription of genes induced in cell proliferation (e.g., *cyclin D1*, *Ciclo-2* and *c-Myc*), angiogenesis (e.g., *VEGF*, *Vascular Endothelial Growth Factor*), and invasion (e.g., *MMP, metalloprotease-9* and *ICAM-1*, *Intercellular Adhesion Molecule-1*). Recent study indicated that survivin assists cancer cells to escape replicative senescence by enhancing telomerase activity.[60] Telomerase is a ribonucleoprotein enzyme that plays a key role in maintaining chromosomal stability and cellular life span.[61] The catalytic subunit of human telomerase (human telomerase reverse transcriptase, hTERT) is expressed in 85% of human cancers, including pancreatic carcinoma cells,[62,63] but usually not in normal cells. Numerous observations indicate that telomere maintenance plays a complex role in human cancer development. Telomere loss limits cell proliferation and serves as a mechanism for tumor suppression. However, sufficient loss of telomere length eventually leads to genomic disarray, which drives tumor formation, through both activating telomerase and generating other mutations necessary for tumor progression.[64]

We found that GEM and 5-FU, both separately and in association, equally repressed telomerase activity in Capan-2 cells.

Since stable overexpression of the anti-apoptotic proteins, such as survivin and bcl-2, in human cancer cells is accompanied by increased levels of telomerase activity,[65] inhibition of the ribonucleoprotein enzyme by 5-FU treatment in Capan-2 cells may be correlated with activation of the apoptosome pathway which involves release of cytochrome *c* from mitochondria, and, by preventing survivin, may be active in counteracting the terminal effectors of apoptosis caspase-3 and caspase-7.[66] How the decrease of telomerase activity is related to GEM treatment remains to be explored, since the drug, in our conditions, affected neither bcl-2 family protein expression nor survivin.

Most current chemotherapy regimens for cancer consist of empirically designed combinations, and several aspects are often overlooked, such as possible metabolic and biological interactions between drugs, scheduling, and different pharmacokinetic profiles. Collectively, these results provide strong molecular evidence in support of the hypothesis that in some pancreatic cancer instance GEM works as an antagonistic agent in combination with 5-FU. These results are also relevant in the context of the clinical course of pancreatic carcinoma, which is characterized by an early propensity to metastasize and a high risk of disease recurrence following resection. If the results of this study are reflected in findings in the clinical setting, adoption of schedules in which GEM is associated to 5-FU may in a proportion of patients, result in sub-optimal anticancer action.

Considering clinical experience, addition of 5-FU to GEM has not replaced GEM alone as the standard of care for patients with pancreatic cancer. Although there are several different ways to modulate 5-FU or the 5-FU schedule, it is unlikely that any of these changes alone will significantly improve therapy of pancreas cancer. More active new agents, such as platinum derivatives cisplatin and oxaliplatin[67] may be considered in combination therapy with GEM.

Increasingly, investigators are recognizing differences in tumor biology, drug metabolism, toxicity, and therapeutic response among different patient populations receiving anticancer agents. Although pharmacogenomic differences may explain some of these disparities, in future rigorous investigation of both genetic and non-genetic differences is important to identify the variables most relevant for optimal selection and dosing of treatment for the individual patient.

References

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics, 2001. CA Cancer J Clin 2001; 51:15-36.
2. Yeo TP, Hruban RH, Leach SD, Wilentz RE, Sohn TA, Kern SE, Iacobuzio-Donahue CA, Maitra A, Goggins M, Canto MI, Abrams RA, Laheru D, Jaffee EM, Hidalgo M, Yeo CJ. Pancreatic cancer. Curr Probl Cancer 2002; 26:176-275.
3. Staley CA, Lee JE, Cleary KR, Abbruzzese JL, Fenoglio CJ, Rich TA, Evans DB. Preoperative chemoradiation, pancreaticoduodenectomy, and intraoperative radiation therapy for adenocarcinoma of the pancreatic head. Am J Surg 1996; 171:118-24.
4. Huang JJ, Yeo CJ, Sohn TA, Lillemoe KD, Sauter PK, Coleman J, Hruban RH, Cameron JL. Quality of life and outcomes after pancreaticoduodenectomy. Ann Surg 2000; 231:890-8.
5. Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D, Greisbach L, Duschnisky R, Schnitzer RJ, Pleven E, Scheiner J. Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. Nature 1957; 179:663-6.
6. Ahlgren JD. Chemotherapy for pancreatic carcinoma. Cancer 1996; 78:654-63.
7. Pasetto LM, Jirillo A, Stefani M, Monfardini S. Old and new drugs in systemic therapy of pancreatic cancer. Crit Rev Oncol Hematol 2004; 49:135-51.
8. Burris IIIrd HA, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: A randomized trial. J Clin Oncol 1997; 15:2403-13.
9. Carmichael J, Fink U, Russell RC, Spittle MF, Harris AL, Spiessi G, Blatter J. Phase II study of gemcitabine in patients with advanced pancreatic cancer. Br J Cancer 1996; 73:101-5.
10. Berlin JD, Alberti DB, Arzoomanian RZ, Feierabend CA, Simon KJ, Binger KA, Marnocha RM, Wilding G. A phase I study of gemcitabine, 5-fluorouracil, and leucovorin in patients with advanced, recurrent and/or metastatic solid tumors. Invest New Drugs 1999; 16:325-30.
11. Berlin JD, Adak S, Vaughn DJ, Flinker D, Blaszkowsky L, Harris JE, Benson IIIrd AB. A phase II study of gemcitabine and 5-fluorouracil in metastatic pancreatic cancer: An Eastern Cooperative Oncology Group Study (*E3296*). Oncology 2000; 58:215-18.
12. Cascinu S, Silva RR, Barni S, Labianca R, Frontini L, Piazza E, Pancera G, Giordani P, Giuliodori L, Pessi MA, Fusco V, Luporini G, Cellerino R, Catalano G. A combination of gemcitabine and 5-fluorouracil in advanced pancreatic cancer, a report from the Italian Group for the Study of Digestive Tract Cancer (GISCAD). Br J Cancer 1999; 80:1595-98.
13. Cascinu S, Silva RR, Barni S, Labianca R, Frontini L, Piazza E, Pancera G, Giordani P, Giuliodori L, Pessi MA, Fusco V, Luporini G, Cellerino R, Catalano G. Grupo de tumores gastrointestinales. Phase II study of gemcitabine, 5-fluorouracil, and leucovorin in patients with pancreatic cancer. Semin Oncol 2001; 28:44-9.
14. Di Costanzo F, Carlini P, Doni L, Massidda B, Mattioli R, Iop A, Barletta E, Moscetti L, Recchia F, Tralongo P, Gasperoni S. Gemcitabine with or without continuous infusion 5-FU in advanced pancreatic cancer: A randomised phase II trial of the Italian oncology group for clinical research (GOIRC). Br J Cancer 2005; 93:185-9.
15. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 1984; 22:27-55.
16. Carbone A, Rodeck U, Mauri FA, Sozzi M, Gaspari F, Smirne C, Prati A, Addeo A, Novarino A, Robecchi A, Bertetto O, Emanuelli G, Bellone G. Human pancreatic carcinoma cells secrete bioactive *interleukin-18* after treatment with *5-fluorouracil*: Implications for anti-tumor immune response. Cancer Biol Ther 2005; 4:231-41.
17. Bellone G, Ferrero D, Carbone A, De Quadros MR, Gramigni C, Prati A, Davidson W, Mioli P, Dughera L, Emanuelli G, Rodeck U. Inhibition of cell survival and invasive potential of colorectal carcinoma cells by the tyrosine kinase inhibitor STI571. Cancer Biol Ther 2004; 3:385-92.
18. Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 2003; 339:62-6.
19. Rasmussen R. Quantification on the LightCycler. In: Meuer S, Wittwer C, Nakagawara K, eds. Rapid Cycle Real-time PCR, Methods and Applications. Heidelberg: Springer Press, 2001:21-34.
20. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002; 30:e36.
21. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 1991; 19:2499.
22. Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin Cancer Res 1999; 5:119-27.
23. Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor-kappaB and IkappaB kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. Cancer 2004; 101:2351-62.
24. Saretzki G. Telomerase inhibition as cancer therapy. Cancer Lett 2003; 194:209-19.
25. Cullinan SA, Moertel CG, Fleming TR, Rubin JR, Krook JE, Everson LK, Windschitl HE, Twito DI, Marschke RF, Foley JF. A comparison of three chemotherapeutic regimens in the treatment of advanced pancreatic and gastric carcinoma. Fluorouracil vs fluorouracil and doxorubicin vs fluorouracil, doxorubicin, and mitomycin. JAMA 1985; 253:2061-67.
26. Storniolo AM, Enas NH, Brown CA, Voi M, Rothenberg ML, Schilsky R. An investigational new drug treatment program for patients with gemcitabine: Results for over 3000 patients with pancreatic carcinoma. Cancer 1999; 85:1261-68.
27. Berlin JD, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson IIIrd AB. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial *E2297*. J Clin Oncol 2002; 20:3270-75.
28. Ricotti L, Tesei A, De Paola F, Ulivi P, Frassineti GL, Milandri C, Amadori D, Zoli W. In vitro schedule-dependent interaction between docetaxel and gemcitabine in human gastric cancer cell lines. Clin Cancer Res 2003; 9:900-5.
29. Zoli W, Ricotti L, Tesei A, Ulivi P, Gasperi Campani A, Fabbri F, Gunelli R, Frassineti GL, Amadori D. Schedule-dependent cytotoxic interaction between epidoxorubicin and gemcitabine in human bladder cancer cells in vitro. Clin Cancer Res 2004; 10:1500-7.
30. Naumann M, Savitskaia N, Eilert C, Schramm A, Kalthoff H, Schmiegel W. Frequent codeletion of *p16/MTS1* and *p15/MTS2* and genetic alterations in *p16/MTS1* in pancreatic tumors. Gastroenterology 1996; 110:1215-24.
31. Kalthoff H, Schmiegel W, Roeder C, Kasche D, Schmidt A, Lauer G, Thiele HG, Honold G, Pantel K, Riethmuller G. *p53* and *K-RAS* alterations in pancreatic epithelial cell lesions. Oncogene 1993; 8:289-98.
32. Berrozpe G, Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the *p53* and *K-ras* genes in pancreatic cancer. Int J Cancer 1994; 58:185-91.
33. Halloran CM, Ghaneh P, Shore S, Greenhalf W, Zumstein L, Wilson D, Neoptolemos JP, Costello E. 5-Fluorouracil or gemcitabine combined with adenoviral-mediated reintroduction of p16INK4A greatly enhanced cytotoxicity in Panc-1 pancreatic adenocarcinoma cells. J Gene Med 2004; 6:514-25.
34. Kimura M, Tagawa M, Takenaga K, Yamaguchi T, Saisho H, Nakagawara A, Sakiyama S. Inability to induce the alteration of tumorigenicity and chemosensitivity of *p53*-null human pancreatic carcinoma cells after the transduction of wild-type *p53* gene. Anticancer Res 1997; 17:873-9.
35. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; 267:1456-62.
36. Hockenbery DM, Oltvai ZN, Yin XM, Milliman CL, Korsmeyer SJ. Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 1993; 75:241-51.
37. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G, Thompson CB. *Bcl-x*, *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 1993; 74:597-608.
38. Schott AF, Apel IJ, Nunez G, Clarke MF. *Bcl-XL* protects cancer cells from *p53*-mediated apoptosis. Oncogene 1995; 11:1389-94.
39. Wang S, Yang D, Lippman ME. Targeting Bcl-2 and Bcl-XL with nonpeptidic small-molecule antagonists. Semin Oncol 2003; 30:133-42.

40. Bettaieb A, Dubrez-Daloz L, Launay S, Plenchette S, Rebe C, Cathelin S, Solary E. *Bcl-2* proteins: Targets and tools for chemosensitisation of tumor cells. Curr Med Chem Anticancer Agents 2003; 3:307-18.
41. Jaattela M. Escaping cell death: Survival proteins in cancer. Exp Cell Res 1999; 248:30-43.
42. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltersdorf T, Reed JC. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998; 58:5315-20.
43. Asanuma K, Tsuji N, Endoh T, Yagihashi A, Watanabe N. Survivin enhances Fas ligand expression via up-regulation of specificity protein 1-mediated gene transcription in colon cancer cells. J Immunol 2004; 172:3922-29.
44. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A. A novel anti-apoptosis gene: Reexpression of survivin messenger *RNA* as a prognosis marker in nonsmall-cell lung cancers. J Clin Oncol 1999; 17:2100-4.
45. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998; 58:5071-4.
46. Nakagawara A. Molecular basis of spontaneous regression of neuroblastoma: Role of neurotrophic signals and genetic abnormalities. Hum Cell 1998; 11:115-24.
47. Kennedy SM, O'Driscoll L, Purcell R, Fitz-Simons N, McDermott EW, Hill AD, O'Higgins NJ, Parkinson M, Linehan R, Clynes M. Prognostic importance of survivin in breast cancer. Br J Cancer 2003; 88:1077-83.
48. Grossman D, McNiff JM, Li F, Altieri DC. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol 1999; 113:1076-81.
49. Zaffaroni N, Daidone MG. Survivin expression and resistance to anticancer treatments: Perspectives for new therapeutic interventions. Drug Resist Updat 2002; 5:65-72.
50. Asanuma K, Moriai R, Yajima T, Yagihashi A, Yamada M, Kobayashi D, Watanabe N. Survivin as a radioresistance factor in pancreatic cancer. Jpn J Cancer Res 2000; 91:1204-9.
51. Olie RA, Simoes-Wust AP, Baumann B, Leech SH, Fabbro D, Stahel RA, Zangemeister-Wittke U. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. Cancer Res 2000; 60:2805-9.
52. Dolcet X, Llobet D, Pallares J, Matias-Guiu X. NFkB in development and progression of human cancer. Virchows Arch 2005; 446:475-82.
53. Sato T, Odagiri H, Ikenaga SK, Maruyama M, Sasaki M. Chemosensitivity of human pancreatic carcinoma cells is enhanced by IkappaBalpha super-repressor. Cancer Sci 2003; 94:467-72.
54. Arlt A, Gehrz A, Muerkoster S, Vorndamm J, Kruse ML, Folsch UR, Schafer H. Role of *NF-kappaB* and *Akt/PI3K* in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death. Oncogene 2003; 22:3243-51.
55. Niu J, Li Z, Peng B, Chiao PJ. Identification of an autoregulatory feedback pathway involving interleukin-1alpha in induction of constitutive NF-kappaB activation in pancreatic cancer cells. J Biol Chem 2004; 279:16452-62.
56. Jung ID, Yang SY, Park CG, Lee KB, Kim JS, Lee SY, Han JW, Lee HW, Lee HY. 5-Fluorouracil inhibits nitric oxide production through the inactivation of IkappaB kinase in stomach cancer cells. Biochem Pharmacol 2002; 64:1439-45.
57. Azuma M, Yamashita T, Aota K, Tamatani T, Sato M. 5-Fluorouracil suppression of NF-KappaB is mediated by the inhibition of IKappab kinase activity in human salivary gland cancer cells. Biochem Biophys Res Commun 2001; 282:292-6.
58. Aota K, Azuma M, Yamashita T, Tamatani T, Motegi K, Ishimaru N, Hayashi Y, Sato M. 5-Fluorouracil induces apoptosis through the suppression of NF-kappaB activity in human salivary gland cancer cells. Biochem Biophys Res Commun 2000; 273:1168-74.
59. Banerjee S, Zhang Y, Ali S, Bhuiyan M, Wang Z, Chiao PJ, Philip PA, Abbruzzese J, Sarkar FH. Molecular evidence for increased antitumor activity of gemcitabine by genistein in vitro and in vivo using an orthotopic model of pancreatic cancer. Cancer Res 2005; 65:9064-72.
60. Endoh T, Tsuji N, Asanuma K, Yagihashi A, Watanabe N. Survivin enhances telomerase activity via up-regulation of specificity *protein 1*- and *c-Myc*-mediated human telomerase reverse transcriptase gene transcription. Exp Cell Res 2005; 305:300-11.
61. Boukamp P, Popp S, Krunic D. Telomere-dependent chromosomal instability. J Investig Dermatol Symp Proc 2005; 10:89-94.
62. Dhaene K, Van Marck E, Parwaresch R. Telomeres, telomerase and cancer: An up-date. Virchows Arch 2000; 437:1-16.
63. Mizumoto K, Tanaka M. Detection of telomerase activity in patients with pancreatic cancer. Methods Mol Med 2005; 103:199-205.
64. Wai LK. Telomeres, telomerase, and tumorigenesis—A review. Med Gen Med 2004; 6:19.
65. Mandal M, Kumar R. Bcl-2 modulates telomerase activity. J Biol Chem 1997; 272:14183-87.
66. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC. A single BIR domain of XIAP sufficient for inhibiting caspases. J Biol Chem 1998; 273:7787-90.
67. Airoldi M, Cattel L, Passera R, Pedani F, Milla P, Zanon C. Gemcitabine and oxaliplatin in patients with pancreatic adenocarcinoma: Clinical and pharmacokinetic data. Pancreas 2006; 32:44-50.